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ORIGINAL PAPER

Genetic variability of VEGF pathway genes in six randomized phase III trials assessing the addition of bevacizumab to standard therapy

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Abstract
Background Despite extensive translational research, no validated biomarkers predictive of bevacizumab treatment outcome have been identified.
Methods We performed a meta-analysis of individual patient data from six randomized phase III trials in colorectal, pancreatic, lung, renal, breast, and gastric cancer to explore the potential relationships between 195 common genetic variants in the vascular endothelial growth factor (VEGF) pathway and bevacizumab treatment outcome.
Results The analysis included 1,402 patients (716 bevacizumab-treated and 686 placebo-treated). Twenty variants were associated ($P < 0.05$) with progression-free survival (PFS) in bevacizumab-treated patients. Of these, 4 variants in *EPAS1* survived correction for multiple testing ($q < 0.05$). Genotype-by-treatment interaction tests revealed that, across these 20 variants, 3 variants in *VEGF-C* (rs12510099), *EPAS1* (rs4953344), and *IL8RA* (rs2234671) were potentially predictive ($P < 0.05$), but not resistant to multiple testing ($q > 0.05$). A weak genotype-by-treatment interaction effect was also observed for rs699946 in *VEGF-A*, whereas Bayesian genewise analysis revealed that genetic variability in *VHL* was associated with PFS in the bevacizumab arm ($q < 0.05$). Variants in *VEGF-A*, *EPAS1*, and *VHL* were located in expression quantitative loci derived from lymphoblastoid cell lines, indicating that they affect the expression levels of their respective gene.
Conclusions This large genetic analysis suggests that variants in *VEGF-A*, *EPAS1*, *IL8RA*, *VHL*, and *VEGF-C* have potential value in predicting bevacizumab treatment outcome across tumor types. Although these associations

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did not survive correction for multiple testing in a genotype-by-interaction analysis, they are among the strongest predictive effects reported to date for genetic variants and bevacizumab efficacy.

Keywords Anti-angiogenesis · Bevacizumab · Treatment outcome · Genetic variant · Predictive and prognostic biomarker

Introduction

Bevacizumab, a humanized monoclonal antibody that binds to vascular endothelial growth factor (VEGF), was the first anti-VEGF-specific drug to be approved in the clinic. The addition of bevacizumab to standard therapy has been shown to significantly improve outcome in patients with metastatic colorectal [1], non-small cell lung [2], breast [3–5], renal [6], and ovarian [7–9] cancer, and recurrent glioblastoma [10]. Although anti-angiogenic drugs have changed clinical practice in several cancers, various clinical challenges remain [11]. For instance, after initial response, adaptive escape mechanisms ultimately lead to disease progression in most patients [12, 13]. There is remarkable heterogeneity in the timing of angiogenic escape, with some patients relapsing almost immediately and others having prolonged periods of stabilized disease [14]. A current challenge, therefore, is to identify and validate markers predictive of bevacizumab treatment outcome. Despite concerted efforts using a broad spectrum of biological sample types [15–17], a validated marker predictive of treatment outcome for bevacizumab therapy has not been identified so far.

As host factors may influence angiogenesis, it is plausible that genetic variability may underlie differences in response to bevacizumab. The characterization of common genetic variability therefore represents a logical focus in bevacizumab biomarker research. Various potential predictive genetic markers have been identified in clinical trials evaluating bevacizumab in several tumor types [18–26] but these analyses, generally using single-trial datasets, are limited by the number of available samples, heterogeneity in the selection of single-nucleotide polymorphisms (SNPs), and lack of understanding of the functional consequences of these genetic markers.

The aim of this study was to identify genetic variants associated with bevacizumab treatment outcome in patients with advanced cancer regardless of tumor type. Although it is not yet clear to what extent markers predictive of treatment response are shared between cancer types, a clear advantage of such an approach is that patients participating in various phase III clinical trials can be combined and much larger patient numbers can be assessed. Here, we pooled patients from six clinical studies in breast, colorectal, pancreatic, gastric, lung, and renal cancers, thereby performing the largest genetic study to date aiming to predict bevacizumab efficacy.

Patients and methods

Patient population

The analysis included samples from patients treated in six randomized phase III trials of bevacizumab in colorectal cancer (NO16966), pancreatic cancer (BO17706; AViTA), advanced or recurrent non-squamous non-small cell lung cancer (BO17704; AVAiL), metastatic renal cell cancer (BO17705; AVOREN), HER2-negative metastatic breast cancer (BO17708; AVADO), and advanced gastric cancer (BO20904; AVAGAST). The designs and primary clinical results (efficacy and safety) for these trials have been published previously [1, 4, 6, 27–29]. Four of the trials met their primary objectives of improving overall survival (OS) and/or progression-free survival (PFS). However, although AViTA and AVAGAST demonstrated improved PFS (secondary end point), the primary end point of OS in these trials did not improve with the addition of bevacizumab to standard chemotherapy. As expected, owing to the fact that different tumor types were assessed, median values and censoring rates for PFS and OS varied considerably between the trials (Table 1).

The genetic analyses were performed on a subset of patients who consented to participate in a genetic substudy, donated a blood sample from which DNA could be successfully extracted and genotyped, and self-reported "white" ethnicity (with the aim of limiting false positives by using an ethnically homogeneous patient population).

Laboratory methods

Peripheral blood samples were collected in K2EDTA Vacutainer tubes (BD, Franklin Lakes, NJ, USA). Germ line DNA was extracted from the precipitated leukocyte cell fraction. Genotyping was performed at the Vesalius Research Center, Leuven, Belgium, with MassARRAY iPLEX Gold (Sequenom, San Diego, CA, USA), as reported previously [23].

We selected SNPs in the following 15 genes involved in the VEGF-A pathway: *VEGF-A*, the *VEGF-A* homologs [placental growth factor (*PlGF*), *VEGF-B*, *VEGF-C*, and *VEGF-D* (also known as c-fos-induced growth factor or *FIGF*)], *VEGF* receptor-1 (*VEGFR-1* or *FLT1*), *VEGF* receptor-2 (*VEGFR-2* or *KDR*) and *VEGF* receptor-3 (*VEGFR-3* or *FLT4*), regulators of hypoxia [hypoxia inducible factor-1α (*HIF1A*), *HIF-2α* (*EPAS1*), factor inhibiting HIF-1A (*FIH1*), von Hippel–Lindau tumor

Table 1 Summary of baseline characteristics and clinical outcomes for patients participating in the clinical studies and the subset of patients consenting to the genetic study

	BO17704 AVAiL	BO17705 AVOREN	BO17706 AViTA	BO17708 AVADO	NO16966	BO20904 AVAGAST	All consented patients, *n*	All patients analyzed, *n*
Primary cancer type	NSCLC	Renal	Pancreatic	Breast	Colorectal	Gastric		
Patients participating in the clinical study, *n*	1,043	649	607	736	1,400	774	5,209	
Patients consenting to the genetic substudy, *n*	119	108	161	350	610	289	1,637	1,402
Bevacizumab, *n* (%)	83 (70)	59 (55)	79 (49)	238 (68)	210 (34)	138 (48)	807 (49)	716
Placebo, *n* (%)	36 (30)	49 (45)	81 (50)	111 (32)	400 (66)	149 (52)	826 (50)	686
Male (%)	71	72	6	0	61	66	51	49
Mean age, years (SD)	57.4 (9.9)	59.7 (10.6)	61.7 (9.6)	54.7 (10.8)	59.6 (11.5)	57.3 (11.3)	58.2 (11.2)	58.7 (10.9)
White (%)	92	99	95	96	85	63	86	100
Median OS, months[a]	13.9	33.2	7.0	28.7	20.9	9.3	17.1	18.1
OS events (%)[a]	67	43	91	63	81	71	73	74
Median PFS, months[a]	6.5	13.6	4.9	8.4	8.8	5.5	7.9	8.2
PFS events (%)[a]	97	89	96	93	96	86	93	94

NSCLC non-small cell lung cancer, *OS* overall survival, *PFS* progression-free survival, *SD* standard deviation

[a] Bevacizumab arm only

suppressor (*VHL*)], and the oxygen sensors [prolyl hydroxylase domain-containing protein 1 (*EGLN2*), 2 (*EGLN1*), and 3 (*EGLN3*)]. A detailed description of how SNPs were selected in these genes has been published previously [23]. Briefly, genomic sequences 5 kb upstream of the translation start site and downstream of the 3′ polyadenylation site of each gene were used to select SNPs from the HapMap database (Phase 2 Public release number 22). Common SNPs with a minor allele frequency $\geq$0.1 and pairwise correlation coefficients (r^2) $\leq$0.8 were selected using the SNP Tagger approach of the Haploview software package [30, 31]. Overall, we selected 211 tagging SNPs in these VEGF pathway genes, as well as 10 genetic variants previously associated with bevacizumab efficacy. Fifteen SNPs known to increase patients' susceptibility to hypertension and thrombosis were also included. These were not analyzed for their effect on bevacizumab treatment outcome, but for correlation with bevacizumab-induced hypertension; results were published recently [32]. After testing for minor allele frequency and the homogeneity of the observed allele frequency (Supplementary Fig. 1), 195 SNPs were included in the analysis (Supplementary Table 1).

Circulating VEGF-A and VEGF-C concentrations were measured in plasma collected from 119 healthy individuals with self-reported Flemish ethnicity for three generations using Human Quantikine ELISA kits (R&D Systems) according to the manufacturer's instructions. Expression and genotype data of 856 lymphoblastoid cell lines derived from healthy female twins of the MuTHER study [33] were used to assess SNP–gene associations in expression quantitative loci (eQTLs) using the Genevar [34] platform.

Statistical methods

Individual patient data were pooled for the meta-analysis. Potential correlations between markers and clinical outcome were assessed in the subset of bevacizumab-treated patients, the subset of placebo-treated patients, and in all patients irrespective of treatment. The end point of primary interest was PFS; OS was a secondary end point. Four covariates were pre-specified for these analyses: geographic region, study, bevacizumab dose, and chemotherapy backbone. Backwards stepwise regression identified additional covariates: Eastern Cooperative Oncology Group (ECOG) performance status (0 vs. 1/2), lactate dehydrogenase level (normal vs. abnormal), baseline serum albumin level ($\leq$29 vs. >29 g/L), and baseline number of lesions ($\leq$2 vs. >2) for PFS and OS. For OS, two additional covariates were identified: alkaline phosphatase level ($\leq$2.5 vs. >2.5 $\times$ upper limit of normal) and gender.

For the single-marker analyses, 195 genetic markers were first tested for single-point association with bevacizumab treatment effect using Cox proportional hazards regression within the subset of patients treated with bevacizumab. Results were then compared with tests of association in the subset of patients not treated with bevacizumab. Genotype-by-treatment interactions were used in the full set to formally test the potential predictive value of candidate markers. Prognostic characteristics were evaluated using tests of association in the full dataset. False discovery rate (FDR) q values were computed to correct for multiple testing, reflecting the exploratory nature of these analyses [35].

For the genewise analysis, associations were tested on a gene-by-gene basis using an empirical Bayesian generalized linear model [36, 37]. This method was originally developed to allow the joint analysis of data from multiple functionally related genes in microarray data, and was implemented to jointly analyze multiple markers from a single gene. The method takes into account the different group sizes, i.e., the different numbers of variants tested in each gene (e.g., >30 variants tested in *EPAS1* vs. 2 or 3 markers tested in most other genes). The implemented method used is available in the R-package as the globaltest. Elastic net analysis was applied to evaluate potential prognostic or predictive effects of marker combinations. A subset of 141 candidate markers with <10 % missing data was subjected to elastic net variable selection [38–40]. Elastic net analysis differs from the above analyses as it allows simultaneous analysis of clinical covariates and markers, thereby identifying the best statistical predictors for a given trait. It encompasses a penalized regression approach that carries advantages of both ridge and lasso penalties, such that it provides shrinkage and variable selection with efficient handling of highly correlated variables (multi-collinearity). Missing genotypes were imputed to the heterozygote genotype (the mode); all variables were standardized, and tuning parameters were selected by two-dimensional tenfold cross-validation. In the interests of sparsity, the partial likelihood deviance was allowed to increase to its highest level such that prediction error was within one standard deviation of the minimum. To obtain a robust estimate of the multivariate model performance, all patients (irrespective of treatment) were stratified by study and randomized to a training or validation set. Elastic net analysis was performed on the training set, and the identified variables were combined into a single continuous classification signature based on the Cox proportional hazards model. The performance of the model was tested using the validation set. Patients were classified into two groups based on the median of the signature, and the two patient subgroups were compared using Kaplan–Meier estimates.

Results

Patient population

The subset of patients included in this genetic substudy comprised 1,402 patients: 716 randomized to bevacizumab and 686 randomized to placebo (Table 1). Only patients who self-reported "white" ethnicity were included in the analysis. Notably, only 60 % of patients participating in the AVAGAST (BO20904) study were from Western Europe, Australia, or North America, whereas in the remaining 5 trials, 88 % of patients were from Western Europe, Australia, or North America, 10 % were from Eastern Europe, and 2 % were from the rest of the world.

Single-marker analyses

Of the 195 variants tested, 20 were associated with PFS in bevacizumab-treated patients at an unadjusted $P < 0.05$ (Table 2; variants are shown in descending order of association strength). Data for all 195 variants tested are listed in Supplementary Table 1. The strongest associations with PFS were seen for variants in *EPAS1* and *VEGF-A* (12 out of 34 *EPAS1* variants and 4 out of 15 *VEGF-A* variants had a $P < 0.05$). The remaining associations were in 2 *VHL* variants, 1 *VEGF-C* and 1 *IL8RA* variant. Notably, 4 variants in *EPAS1* (rs4145836, rs11689649, rs7594278, and rs1374749), of which rs4145836 was most significantly associated with PFS [hazard ratio (HR) 0.69; 95 % confidence interval (CI) 0.58–0.83; $P = 0.0001$], also survived correction for multiple testing ($q < 0.05$ using FDR correction).

Genotype-by-treatment interaction tests indicated a potential predictive effect for 3 of these 20 variants: rs12510099 in *VEGF-C* (interaction $P = 0.0311$), rs4953344 in *EPAS1* (interaction $P = 0.0454$), and rs2234671 in *IL8RA* (interaction $P = 0.0492$). None of these SNPs survived correction for multiple testing ($q > 0.05$). Kaplan–Meier curves for these individual SNPs are shown in Fig. 1. Forest plots displaying HRs in the individual studies are presented in Fig. 2. One additional variant (rs699946 in the *VEGF-A* promoter) showed a trend toward a potential predictive interaction effect (interaction $P = 0.0907$; Table 2). The allelic HR for this SNP was 1.27 (95 % CI 1.08–1.49; $P = 0.0034$), whereas no effect was seen in placebo-treated patients ($P = 0.9110$). The nearby rs699947 SNP in *VEGF-A*, which was previously identified as a predictor of bevacizumab treatment outcome in the E2100 trial in breast cancer [20], was not associated with bevacizumab treatment outcome in our analysis.

Although our primary end point of interest was PFS, similar analyses were performed for the secondary end point, OS. Eight of the 195 variants showed a potential

Table 2 Markers with $P < 0.05$ in association testing of 195 markers against PFS in white patients receiving bevacizumab, with corresponding data in placebo-treated patients. The four variants with the lowest genotype-by-treatment interaction *P*-value ($P < 0.1$) are marked in bold

Marker	Chr	All white			Bevacizumab				Placebo	Genotype-by-treatment interaction *P*	Gene
		Position	MAF	HWE *P*	*N*	HR	95 % CI	*P* value	*P* value		
rs4145836	2	46595363	0.13	0.96	641	0.69	0.58–0.83	1.00E−04	0.0059	0.7874	*EPAS1*
rs11689649	2	46617118	0.50	0.79	485	1.28	1.12–1.47	3.00E−04	0.5098	0.1521	*EPAS1*
rs7594278	2	46604593	0.47	0.61	656	1.22	1.09–1.37	6.00E−04	0.1320	0.3731	*EPAS1*
rs1374749	2	46596433	0.48	0.56	660	0.82	0.73–0.92	8.00E−04	0.3062	0.1659	*EPAS1*
rs6753127	2	46597296	0.08	0.06	661	0.73	0.59–0.89	0.0025	0.3337	0.2381	*EPAS1*
rs3768730	2	46592524	0.47	0.80	463	1.25	1.08–1.44	0.0028	0.2831	0.4392	*EPAS1*
rs699946	**6**	**43732669**	**0.18**	**0.30**	**542**	**1.27**	**1.08–1.49**	**0.0034**	**0.9110**	**0.0907**	***VEGF-A***
rs1678607	3	10188428	0.13	0.28	659	0.78	0.66–0.93	0.0051	0.2200	0.4150	*VHL*
rs833058	6	43731854	0.37	0.88	660	1.18	1.05–1.32	0.0053	0.5404	0.2328	*VEGF-A*
rs7565341	2	46599030	0.41	0.31	654	0.85	0.76–0.95	0.0055	0.3361	0.2804	*EPAS1*
rs12510099	**4**	**177602953**	**0.09**	**0.94**	**643**	**0.73**	**0.58–0.91**	**0.0060**	**0.9266**	**0.0311**	***VEGF-C***
rs3025030	6	43750587	0.14	0.12	636	1.22	1.04–1.43	0.0124	0.4816	0.4143	*VEGF-A*
rs3025039	6	43752536	0.15	0.31	703	1.21	1.04–1.40	0.0135	0.8285	0.2277	*VEGF-A*
rs2881324	2	46576894	0.49	0.27	327	0.82	0.69–0.96	0.0153	0.1234	0.8957	*EPAS1*
rs1562452	2	46580444	0.50	0.59	661	0.87	0.77–0.97	0.0155	0.0232	0.8606	*EPAS1*
rs1642742	3	10191943	0.32	0.55	650	0.86	0.77–0.97	0.0163	0.9199	0.1540	*VHL*
rs4953344	**2**	**46552458**	**0.16**	**0.43**	**640**	**0.83**	**0.71–0.97**	**0.0165**	**0.4763**	**0.0454**	***EPAS1***
rs13409493	2	46588488	0.12	0.59	587	0.82	0.68–0.98	0.0338	0.5773	0.3727	*EPAS1*
rs2234671	**2**	**219029108**	**0.05**	**0.28**	**695**	**1.33**	**1.02–1.73**	**0.0376**	**0.4303**	**0.0492**	***IL8RA***
rs2121267	2	46549389	0.45	0.77	661	1.13	1.01–1.26	0.0381	0.8354	0.2354	*EPAS1*

Chr chromosome, *CI* confidence interval, *N* number of patients, *MAF* minor allele frequency, *HR* hazard ratio, *HWE P* Hardy–Weinberg *P* value

association ($P < 0.05$) with OS in bevacizumab-treated patients (Supplementary Table 2). Three of these variants were located in *VEGFR-1* (*FLT1*). The most distinct association (HR 1.41, 95 % CI 1.15–1.72; $P = 0.001$) was for rs12505758, which is one of the 26 *VEGFR-2* (*KDR*) variants tested. However, none of the 8 variants surpassed FDR correction. Genotype-by-treatment interaction tests suggested a potential predictive effect on OS for rs7987649 in *VEGFR-1* (interaction $P = 0.0062$) and rs12505758 in *VEGFR-2* (interaction $P = 0.0165$; Supplementary Table 2).

We also assessed the effect of these 195 variants on PFS irrespective of treatment arm. Analysis of PFS data collected from all 1,402 patients treated with either bevacizumab or placebo revealed 25 variants exhibiting $P < 0.05$. Twelve of these variants were located in *EPAS1* (Supplementary Table 3). Similar to the bevacizumab-treated arm, rs4145836 in *EPAS1* was most significantly associated with PFS (Fig. 3a; allelic HR 0.74; 95 % CI 0.65–0.84; $P = 2.2 \times 10^{-6}$) and survived correction for multiple testing. In 5 of 6 trials, the HRs for rs4145836 consistently pointed in the same direction, indicating that the prognostic effect of rs4145836 was consistent across tumor types (Fig. 3b). Overall, 5 markers in *EPAS1* (rs4145836, rs6712143, rs6715787, rs1562452, and rs7594278) were associated with PFS after correcting for multiple testing ($q < 0.05$ using FDR correction). Similar analyses for OS in all 1,402 patients revealed that 15 out of 195 variants were associated with OS at $P < 0.05$. Six of these were in *EPAS1*, but none of them surpassed the multiple testing threshold. Notably, the association between rs4145836 in *EPAS1* and OS (HR 0.81; 95 % CI 0.71–0.94; $P = 0.0045$) was in the same direction as observed for PFS.

Genewise analyses and elastic net analyses

Next, we complemented these single-variant analyses with a Bayesian genewise association analysis for PFS. By considering all variants located in a specific gene simultaneously, this analysis explored whether the combined effects of individual variants were associated with PFS either in the bevacizumab-treated group or in the overall patient population. Eighteen genes containing 2 or more variants were considered for this analysis. After correction for multiple testing using the FDR approach, 2 genes were associated with PFS ($q < 0.05$) in the bevacizumab arm: *EPAS1* (34 variants) and *VHL* (2 variants). In the entire population of 1,402 patients, only *EPAS1* had an FDR-adjusted $q < 0.05$ for PFS. In a similar genewise analysis

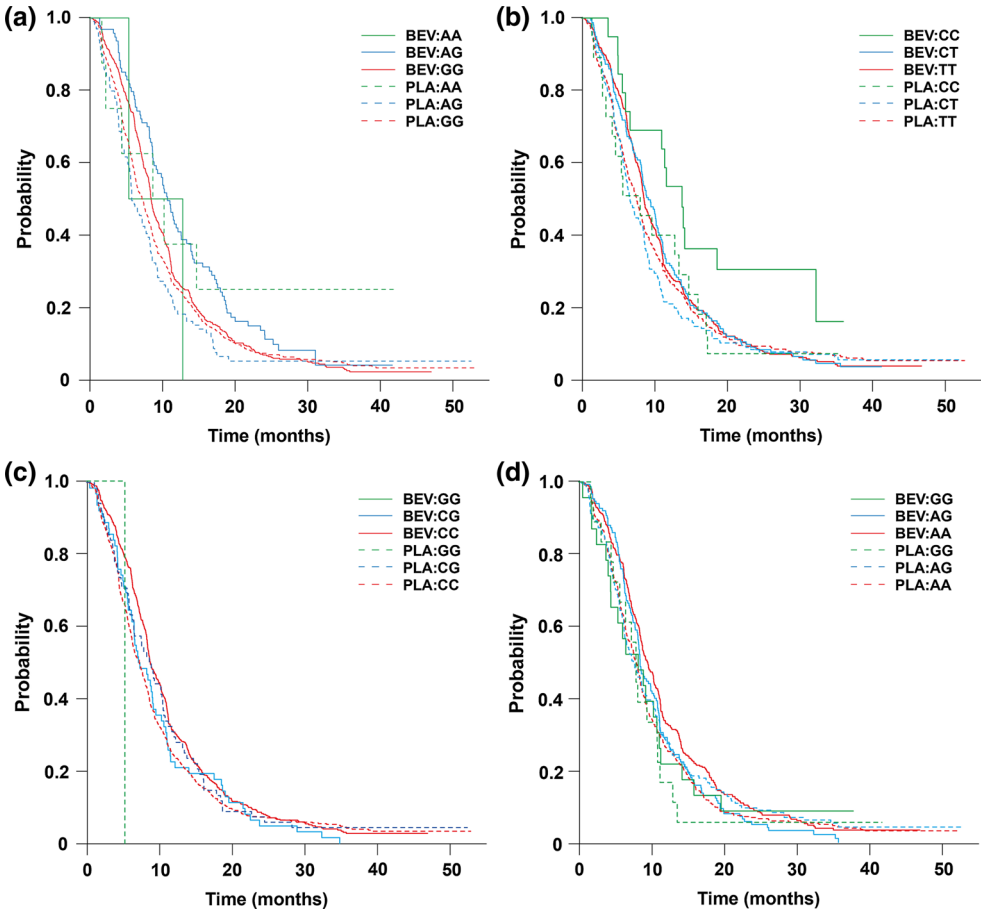

Fig. 1 Kaplan–Meier curves for progression-free survival stratified by treatment arm and single-nucleotide polymorphisms. **a** rs12510099 in *VEGF-C*, **b** rs4953344 in *EPAS1*, **c** rs2234671 in *IL8RA*, and **d** rs699946 in *VEGF-A*

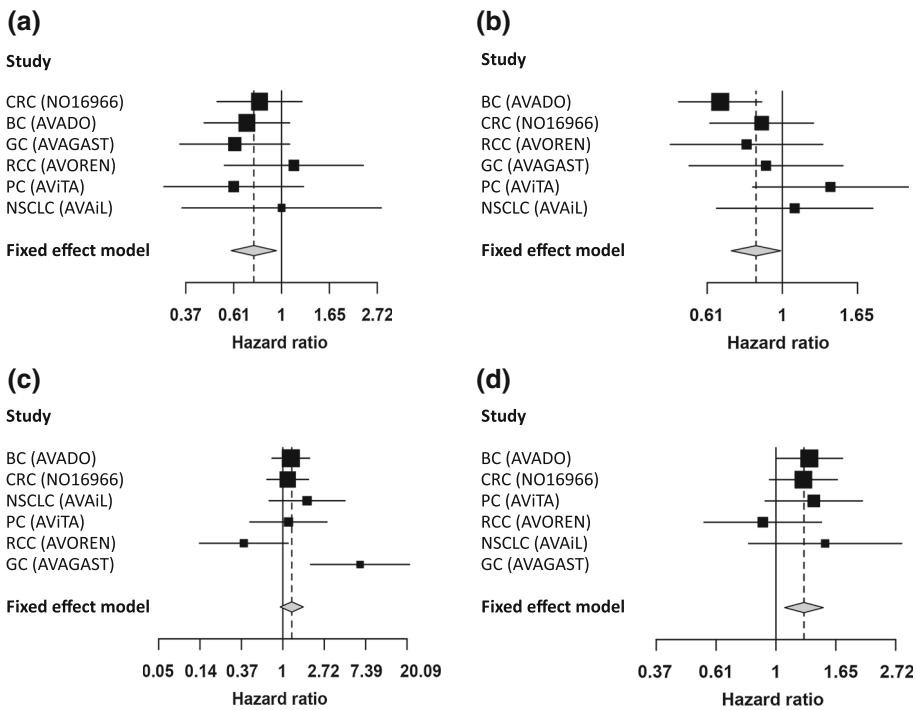

Fig. 2 Forest plots showing the association of progression-free survival with **a** rs12510099 in *VEGF-C*, **b** rs4953344 in *EPAS1*, **c** rs2234671 in *IL8RA*, and **d** rs699946 in *VEGF-A*. *CRC* colorectal cancer, *BC* breast cancer, *GC* gastric cancer, *RCC* renal cell cancer, *PC* pancreatic cancer, *NSCLC* non-squamous non-small cell lung cancer

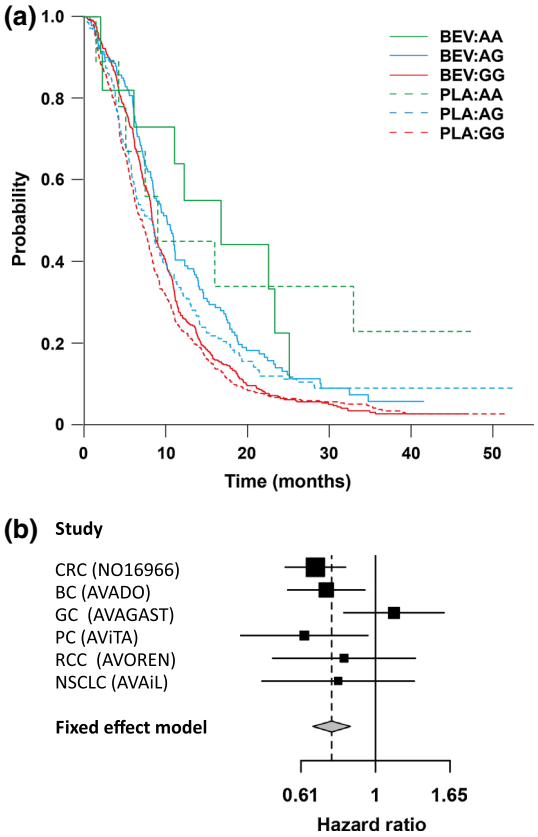

Fig. 3 **a** Kaplan–Meier curves for progression-free survival stratified by treatment arm and rs4145836 genotypes. **b** Forest plot showing the association of rs4145836 in *EPAS1* with progression-free survival. *CRC* colorectal cancer, *BC* breast cancer, *GC* gastric cancer, *RCC* renal cell cancer, *PC* pancreatic cancer, *NSCLC* non-squamous non-small cell lung cancer

for OS, none of the genes had an FDR-adjusted $q < 0.05$ for OS in either the bevacizumab-treated subset or the combination of both treatment arms.

Given the consistent prognostic effect of *EPAS1* variants observed in the univariate and genewise analyses, a multivariate elastic net analysis was undertaken to identify a combination of genetic and clinical variables prognostic for clinical outcome. With respect to PFS, 4 clinical covariates (region, concomitant gemcitabine, ECOG performance status, and trial) and two genetic variants [rs4145836 in *EPAS1* and rs3034659, the *+4422(AC)11-14* repeat in *VEGFR-2*] were identified as a signature in the training set (Supplementary Table 4a). Figure 4a shows the Kaplan–Meier curve in the validation set when classified by the median of this signature. The HR comparing the "above median" versus "below median" subgroups revealed a substantial and significant difference in the overall population (HR 0.57; 95 % CI 0.48–0.67; $P = 2.9 \times 10^{-12}$) and within both individual treatment arms (bevacizumab: HR 0.59; 95 % CI 0.47–0.73; $P = 2.2 \times 10^{-6}$; placebo: HR 0.54; 95 % CI 0.43–0.68; $P = 1.4 \times 10^{-7}$). The performance of the PFS signature was also investigated with respect to OS in the validation set (Fig. 4b) and demonstrated a similar strong effect (HR 0.43; 95 %CI 0.36–0.52).

When using the elastic net analysis for the identification of predictors for OS, 9 clinical covariates were identified in the training set as well as 8 genetic variants (rs1042886 in *PlGF*, rs12888409 in *HIF-1A*, rs1870377 and rs2125489 in *VEGFR-2*, rs2281827 and rs9508021 in *VEGFR-1*, and rs4145836 and rs9973653 in *EPAS1*; Supplementary Table 4b). Notably, bevacizumab treatment was again not identified as one of the 9 clinical covariates. When this OS signature was evaluated in the validation set, the HR for OS comparing above versus below the median was substantial and significant (HR 0.38; 95 % CI 0.32–0.46; $P < 2 \times 10^{-16}$; Supplementary Fig. 2). The performance of the OS signature was also investigated with respect to PFS in the validation set and showed a similarly strong effect (HR 0.52; 95 % CI 0.44–0.60; $P = 3.3 \times 10^{-16}$).

Functional effects of variants predictive of bevacizumab outcome

To test whether any of these significant SNPs also affect mRNA expression of their respective genes, we assessed whether they were located in expression quantitative trait loci (eQTL), as identified by Genevar [34]. Using mRNA expression data from 856 lymphoblastoid cell lines [33], we confirmed that rs699946 in *VEGF-A* was located in an eQTL ($P = 0.0161$; Fig. 5a). In particular, rs699946 AA carriers exhibited a 6 and 12 % reduction in mRNA expression compared to, respectively, rs699946 GA and GG carriers. In contrast, rs2234671 and rs12510099 were not located in an eQTL associated from *IL8RA* or *VEGF-C*. Several of the *EPAS1* SNPs did, however, locate in an eQTL from *EPAS1*. In particular, although the predictive rs4953344 variant in *EPAS1* itself was not genotyped, the SNP with the highest r^2 value to rs4953344, i.e., rs3768727, was significantly associated with *EPAS1* expression ($P = 0.0437$; Fig. 5b). Also, the minor rs13409493 T-allele, which was predictive in the meta-analysis and correlated with improved PFS (Table 2), was significantly associated with increased *EPAS1* mRNA expression ($P = 0.0326$; Fig. 5b), whereas the rs4145836 variant, which was associated with a prognostic effect, was not in an *EPAS1* eQTL. On the other hand, we confirmed that rs1678607 and rs1642742 in *VHL* were both located in an eQTL significantly determining *VHL* mRNA expression ($P = 4.77 \times 10^{-8}$ and $P = 2.58 \times 10^{-4}$, respectively; Fig. 5c). In particular, minor alleles of both SNPs strongly correlated with increased *VHL* mRNA expression.

We observed a similar correlation between rs699946 and circulating plasma VEGF-A concentration in healthy

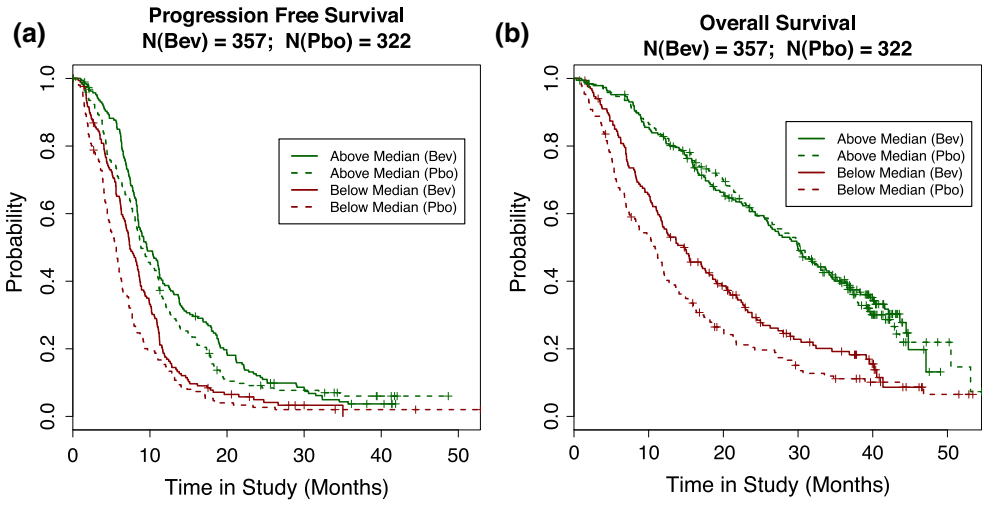

Fig. 4 Kaplan–Meier curves in the validation set classified based on the median of the signature for **a** PFS and **b** OS. The model was derived for PFS in a training set comprising half of the bevacizumab-treated and half of the placebo-treated patients

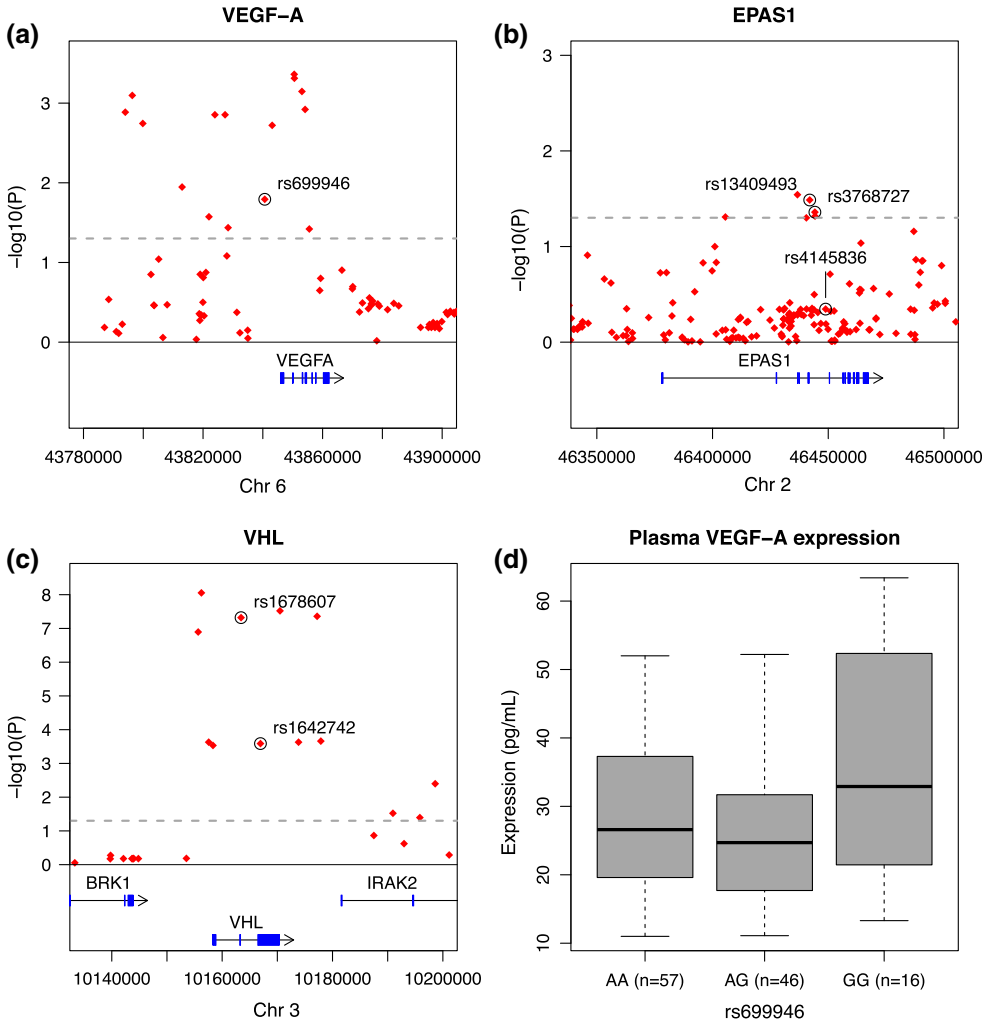

Fig. 5 Regional plots from the eQTL analysis in 856 lymphoblastoid cell lines of healthy female twins of the MuTHER study for **a** *VEGF-A*, **b** *EPAS1,* and **c** *VHL*. On the *X*-axis, the chromosomal positions of the SNPs are shown; on the *Y*-axis, the $-\log_{10} P$ value obtained for the eQTL analysis between the SNPs against mRNA expression of their respective genes is shown. Variants identified in this study, or their closest proxies genotyped in the MuTHER study, are indicated in the individual plots. **d** The correlation of VEGF plasma levels with SNP rs699946 genotypes. *BEV* bevacizumab, *OS* overall survival, *PFS* progression-free survival, *PLA* placebo

individuals ($P = 0.006$; Fig. 5d) but not between rs12510099 and plasma VEGF-C concentration. EPAS1, VHL, and IL8RA were not detectable in plasma as these proteins are intracellular or membrane-bound.

Discussion

The most significant finding of this study is the identification of 3 genetic variants in *VEGF-C*, *EPAS1*, and *IL8RA* that were predictive of bevacizumab treatment outcome. The effects of these variants were significant ($P < 0.05$) when assessing PFS in bevacizumab-treated patients only, as well as in a genotype-by-treatment interaction analysis. One additional variant in *VEGF*-A was also predictive in bevacizumab-treated patients but failed to reach significance in the interaction analysis ($P = 0.091$). This variant could nevertheless be considered as an additional variant potentially predictive of bevacizumab treatment outcome, as it significantly affected VEGF expression. Two variants, rs12505758 in *VEGFR-2* and rs7987649 in *VEGFR-1*, were also predictive for OS. The interpretation of OS data was, however, more difficult as crossover events might have affected the OS analysis. It should be noted that none of the treatment-by-interaction effects for PFS or OS remained statistically significant after FDR correction for testing 195 variants. Replication in additional studies is therefore needed before considering these markers as true predictors of bevacizumab treatment outcome across tumor types.

A correlation between rs2234671 in *IL8RA* (*CXCR1*) and treatment outcome has previously been described in a single-arm study of 132 patients receiving bevacizumab for colorectal cancer [22]. Wild-type CC carriers were characterized by increased response rates and prolonged PFS. In our much larger cohort of 716 bevacizumab-treated patients, similar effects on PFS were noticed, but importantly, rs2234671 was also significant in the treatment-by-interaction analysis including 686 patients receiving placebo. The rs2234671 variant represents a non-synonymous SNP in exon 1 of *CXCR1*, which tags a large linkage disequilibrium block across *CXCR1*. Although rs2234671 did not correlate with altered *CXCR1* mRNA expression in Genevar, it might still reduce IL8 signaling at the protein level by introducing an amino acid substitution (S276T) at a conserved position. Notably, altered expression of *CXCR1* has previously been reported to regulate angiogenesis independently of VEGF-A and to activate a tumor-specific immune response by attracting leukocytes, potentially suggesting mechanisms by which this variant might affect bevacizumab treatment outcome [41].

The rs699946 variant, which is located in the *VEGF-A* promoter, was previously identified as a marker of treatment outcome in patients receiving bevacizumab for neovascular age-related macular degeneration [42]. In the present study, AA carriers of rs699946 exhibited an improved PFS after bevacizumab treatment compared with placebo-treated patients. When stratifying VEGF plasma concentrations from healthy subjects according to rs699946, we noticed that VEGF concentrations were lower in AA than GG carriers, an effect that was confirmed at the mRNA level in Genevar. On the other hand, rs12510099 in *VEGF-C* has not yet been studied and since it did also not affect *VEGF-C* expression, its role in predicting bevacizumab treatment outcome is currently unclear. Finally, we also identified 2 variants in *VHL* that were associated with bevacizumab treatment outcome at $P < 0.05$. In the genewise analysis, the combined effects of both these variants were significant even at an FDR-adjusted $q < 0.05$ for PFS. Minor alleles of both variants also correlated with increased *VHL* mRNA expression in Genevar and were associated with improved PFS after bevacizumab.

Another intriguing finding is that, although several variants in *EPAS1* were significant ($P < 0.05$) when assessing PFS in bevacizumab-treated patients only, one variant in *EPAS1* exerted a strong prognostic rather than predictive effect on PFS. The prognostic effect of the rs4145836 variant resisted correction for multiple testing and was also retrieved in the multivariate signature identified by elastic net analyses. EPAS1 (or HIF-2α) is an oxygen-sensitive transcription factor that allows adaptation of cells to hypoxic environments [43]. It has a well-established role during angiogenesis [44–46], but recent evidence also implicates this gene as a key mediator of the metabolic adaption of tumors and the infiltration of inflammatory cells into the tumor microenvironment [47]. Previous studies revealed, for instance, that EPAS1, as assessed by immunohistochemistry, is present at increased levels in some tumors and that these patients have significantly decreased survival compared with patients whose tumor samples have undetectable levels of EPAS1 [48]. Furthermore, *EPAS1* expression appears to be upregulated in the surrounding stroma, in particular in tumor-associated macrophages, as opposed to the tumor cells [49]. These elevated expression levels of *EPAS1* in tumor-associated macrophages correspond directly with clinical severity of many different human cancers [50]. Interestingly, several genome-wide studies conducted in populations biologically adapted to living at high altitude, such as the Tibetan population, revealed *EPAS1* variants that were under strong genetic selection and responsible for high-altitude adaptations [51–53]. Some of the variants identified in the present study were in high linkage disequilibrium with these variants supporting the notion that they affect *EPAS1* expression or function. Genevar analysis indeed confirmed that several of these SNPs were highly significantly

correlated with *EPAS1* mRNA expression levels. Additional fine-mapping studies should be conducted to identify the causal variant(s) in *EPAS1* contributing to these phenotypes. With respect to the latter, carriers of *EPAS1* genotypes that correlated increased EPAS1 expression exhibited prolonged PFS.

Finally, using elastic net analyses to identify genetic variants and clinical variables significantly contributing to clinical outcome, we observed that bevacizumab treatment did not significantly contribute to the regression model, whereas, surprisingly, several of the genetic variants did contribute. The lack of an association with bevacizumab treatment can probably be explained by the fact that bevacizumab exerts very minimal therapeutic effects in some of the cancer types included in the meta-analysis, whereas, on the other hand, the fact that variants in angiogenesis-related genes were identified as prognostic factors across various cancer types is noteworthy.

With respect to the statistical analysis, we chose to apply a pooled meta-analysis of individual patient data rather than a random-effects meta-analysis. Although the latter model is intuitively appealing, there were insufficient clinical studies to precisely model the between-studies variance. Another potential weakness of the study is the lack of a replication set. We decided, however, to combine all available data into a single analysis to maximize statistical power. Moreover, in the case of the association between PFS and rs4145836, the effect size estimated from pooling individual patient data was HR 0.74, very close to that derived from the fixed-effects meta-analysis of summary statistics (HR 0.75), suggesting that the association was not attributable to patient stratification. Finally, since we selected 195 variants that were in low LD with each other, we did not perform a haplotype-based analysis, as has previously been performed, for instance, for the *VEGF-A* promoter [54, 55].

In conclusion, by performing a meta-analysis of individual data from more than 1,400 patients across 6 different tumor types, we identified several potentially predictive markers of bevacizumab treatment outcome that also functionally affected the expression levels of their target genes. We anticipate that these findings will contribute to ongoing efforts aimed at identifying markers that predict which patients will benefit most from bevacizumab therapy.

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Conflict of interest Sanne de Haas and Paul Delmar are employees of F. Hoffmann-La Roche, Basel, Switzerland. Aruna T. Bansal is a paid consultant of F. Hoffmann-La Roche. Eric Van Cutsem, Diether Lambrechts, and Peter Carmeliet have received research funding from F. Hoffmann-La Roche related to research into biomarkers for bevacizumab. David Miles has received honoraria from F. Hoffmann-La Roche for advisory boards and speaker engagements. Celine Pallaud is a former employee of F. Hoffmann-La Roche, Basel, Switzerland. Stefan Scherer is a former employee of Genentech. The remaining authors have declared no potential conflict of interest.

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